THE SIGNIFICANCE OF PLATE COUNTS OF SOIL FUNGI AND THE DETECTION OF THEIR MYCELIA

Toshi SAITÔ

THE elucidation of the fungus population in the soil involves a question of importance, whether it consists of vegetative mycelia leading an active life in the soil or of dormant spores. It must regretfully be said, however, that it is not possible to know which of colonies that are developed on a plate inoculated with the soil are derived either from mycelia or from spores. In reality the plate method is far from being an appropriate approach to the fungus population of the soil. On the other hand, in the soil itself the mycelia of a variety of fungi are continuously extending, much entangled and not markedly differentiated from one another, and consequently preclude qualitative and quantitative estimations. By the direct microscopic examination and the contact slide method the specific determination is not possible. In this note the significance of the plate counts of soil fungi and related problems are dealt with.

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PRINCIPAL DEFECTS OF THE PLATE METHOD

In the plate method of isolating and counting soil fungi, the soil is first shaken vigorously with water with the aim of separating fungus mycelia and spores from soil particles. Nevertheless, as will clearly be seen from the facts mentioned below, the mycelium, which is split into pieces by the shaking, is as a rule attached firmly to soil particles, often penetrating into their crevices, and its longer pieces, in particular, tend to remain on rapidly settling coarse soil particles without being brought into suspension, a portion of which is plated out. Only fragments of hyphae, too short to develop when plated out (see below), may be brought into suspension. Hence the plating technique gives no clear picture, so far as the distribution of vegetative mycelia in the soil is concerned, and *the plate counts refer principally to the occurrence of fungus spores in the soil, whose production varies from species to species a great deal.* As a consequence, moreover, *less easily sporulating fungi such as Basidiomycetes are apt to be ignored.*

The spores themselves which give rise to colonies on the plate are not necessarily autochthonous. Thus, they fall into three categories: that is to say, first, those

having been borne on active mycelia in the soil and fallen just by shaking; secondly, those which are also autochthonous but have previously been set free from the mycelia and still resting without germinating; and thirdly, the spores of allochthonous fungi which have no power to develop in soil environment.

Microscopic findings of soil suspension

In the first place the soil deliberately enriched with fungi was used as material. A sterilised garden soil moistened with a nutrient solution containing glucose and peptone was inoculated with the spores of *Penicillium chrysogenum*, *Aspergillus glaucus* and *Trichoderma* sp. separately. After incubating at 27°C. for three days, the soil is overgrown with the mould so that its surface is coloured greyish white. This mouldy soil was shaken with water five times as much as the soil for five minutes and, after letting it stand for a while, the supernatant liquid was examined under the microscope. The spores are seen abundantly, but the mycelia, mostly being in short fragments, are only rarely found. The sedimented soil, on the other hand, shows on microscopic examination numerous long hyphae twined round the soil particles.

In marked contrast with the former are natural soils. The supernatant liquid is found to be almost free from mycelia, whilst spores are noticed in it. Even in the sedimented soil only a relatively small number of pieces of mycelia are seen, varying in number according to the type of the soil. Among them are winding hyphae which are as long as several mm. and adhere to the soil particles.

Inability of development of short mycelial fragments brought into suspension

Based on observations of the first stage of development of colonies on agar plates inoculated with the supernatant suspension and the rough sediment separately, the following conclusions may be drawn:

1. It appears that the short fragments of mycelia, which are formed by shaking and sometimes met with in the supernatant suspension left after quickly settling larger soil particles have sunk, are on the whole incapable of developing any further.

2. Therefore, the mycelium is almost excluded from the plate count.

3. The long pieces of mycelium twined round the sedimented larger soil particles are likely to give rise to colonies occasionally.

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Fungal filaments microscopically recognised in natural soils

Forest and garden soils are relatively rich in mycelia compared with grassland and paddy-field soils. In the forest soil fungus hyphae often twine themselves round plant residues. Fruiting organs are rarely found except on organic residues.

On direct microscopic examination of such natural soils, three types of fungal filaments are usually noticed: namely, in the first place, non-septate, broad phycomycetous hyphae; secondly, the most widespread, much-branched, septate, fine hyphae; and lastly, dark-coloured hyphae. Owing to the close contact with soil particles, these hyphae, unlike those developed on artificial media, are of more or less irregular form and devoid of smooth surfaces. Apart from spores possessing peculiar forms, such as of *Fusarium* and *Helminthosporium*, the distinction of spores distributed in the soil is not possible.

Cultivation of mycelia picked out of the soil

An attempt was made to pick out of the soil a piece of mycelium and cultivate it on an agar medium. A small amount of the soil moistened with water is spread in a thin layer on acid glucose-peptone agar by the use of a glass rod with a crooked end covered with a rubber tube. By means of a metal cap which is attached to the objective of the microscope and provided with a boring tube—1 or 2 mm. in diameter—at the centre, an agar disk is cut by lowering the tube of the microscope, when a suitable mycelium is found through the tube of the metal cap. From the agar disk placed on a fresh agar plate, the mycelial piece alone is picked out, and then transferred to another agar plate and incubated. Needless to say, special skill and care are here required to avoid contamination. And to find out an appropriate piece of mycelium in the soil is often difficult, and, as a consequence, this method of isolating mycelia directly from the soil is not always successful. But, as will be seen from the following examples, it throws light to some extent on the occurrence of fungal mycelia in the soil.

Cultures from isolated mycelia in comparison with colonies developed on plates

By the above method I could reveal the presence of the mycelia of *Trichoderma lignorum* in litter and of *Penicillium expansum* in humus soil of a plantation of *Chamaecyparis obtusa* with a undergrowth of *Sasa* sp. associated with *Ardisia japonica* in the suburbs of Sendai. On direct microscopic examination of this soil three types of fungal mycelia are distinguished, they occurring in abundance particularly in

the litter:

1. Predominating brownish black hyphae 3.0–3.5 μ in width.
2. Hyaline, septate, broad hyphae 3.0–4.0 μ in width with warty surfaces.
3. Hyaline, septate, fine hyphae.

Of these three types the first two fail to develop on agar media, such as acid glucose-peptone agar, Czapek's solution agar and litter-decoction agar. *Trichoderma lignorum* and *Penicillium expansum* whose mycelia were isolated directly are of the third type. Fungus colonies developed on plates inoculated with the same soil consist actually of *Trichoderma* and *Penicillium*.

In the shade of a tree of *Cercidiphyllum japonicum* growing in the garden of our laboratory, *Penicillium janthinellium* and *Mucor hiemalis* were grown from mycelia picked out of the soil, both — bearing fruiting bodies — from the litter layer and the former alone from the underlying humus soil. Plate counts of the same soil give by far the greatest percentage of *Penicillium janthinellium*, associated with *Mucor hiemalis*, *M. mucedo*, *Cladosporium herbarium*, *Trichoderma lignorum*, *Fusarium* sp. and *Penicillium* sp.

Lathyrus maritimus is common on sandy beach in the neighbourhood of Sendai. *Phoma* sp. was cultivated from mycelia interlaced with its roots. But, by the plating technique not only *Phoma* but also *Penicillium* spp., *Aspergillus niger*, *Macrosporium* sp. and *Dematium* sp. were isolated from the rhizosphere of this plant.

Review of Waksman's direct inoculation method

Waksman* has put forward "a direct inoculation method" to prove the presence of fungal mycelia in the soil. It consists in placing a lump of soil about 1 cm. in diameter in the middle of a plate of Czapek's solution agar, and incubating at 20–22°C. for 24 hours. Mycelia radiating into the surrounding medium from the lump of soil within this space of time, he says, ought to be derived from active mycelia present in the soil, for the time of incubation at that temperature is not sufficient for the development of mycelium from spores. This idea is based on his observations with a mass of mycelium, on one hand, and a single spore, on the other, placed on the same medium instead of the soil.

However, this was not the case with my experimentation. It is to be noted that fungal mycelia in general are distributed in the soil not in compact masses as colonies grown on culture media, but in loose wefts twining themselves round soil particles. Although even the comparatively slowly growing *Penicillia* really extend

* Waksman, S. A. (1916): Do fungi actually live in the soil and produce mycelium? *Science*, N. S., **44**, 320–322.

filaments in 24 hours at the same temperature as above from a compact mass no less than about 1 sq. mm.; if a loose mass of mycelium just as seen in the soil is used as an inoculum, no growth whatever is observed with the naked eye within that time. Their spores barely give rise to microscopic germ tubes, without forming conspicuous elongated hyphae. Unlike *Penicillia*, mucoraceous fungi, such as *Mucor mucedo* and *Rhizopus nigricans*, do show a development not only from a small amount of loose mycelia but also from a single spore, though CZAPEK's solution is said to be unsuitable for the growth of Mucorales.

Three samples of garden soil showed a development of colonies of exclusively mucoraceous fungi from the lumps of soil, according to WAKSMAN's direct inoculation method—viz. two species of *Mucor*, a species of *Rhizopus* and a species of *Zygorhynchus*. Apart from septate, fine hyphae, however, none of the non-septate, broad hyphae characteristic of the mucoraceous fungi could be found in these soils even by careful direct microscopic examination; therefore, the mycelia developed from the lumps of soil should be derived from spores.

SPECIFIC GRAVITIES OF FUNGAL MYCELIA

In connection with the problem whether the fungal mycelium is easily brought into suspension, the specific gravities of mycelia were determined of three species of fungi, *Penicillium digitatum* isolated from a rotten orange, and *Trichoderma koningi* and *Alternaria* sp. both from the soil.

A bit of the sporulating mycelial mat developed on the surface of a glucose-peptone solution was placed in a pyknometer. Prior to it, the culture solution attached to the lower surface of the mat had been removed with filter paper. The pyknometer with the mycelium was first half filled with water, evacuated at the pressure of 4 to 5 mm. Hg in order to drive air bubbles from the mycelium, and then entirely filled with water. The results are set out in Table 1.

The spores of higher fungi have been reported by BULLER* to have specific gravities ranging from 1.02 to 1.21.

Table 1. The specific gravities of mycelia.

Penicillium digitatum	1.121
Trichoderma koningi	1.131
Alternaria sp.	1.131

SUMMARY

1. The plate count refers in the main to the distribution of fungal spores

* BULLER, A. H. R. (1909) : *Researches of Fungi.* Vol. 1, p. 153.

in the soil, whose production varies from species to species and part of which is even merely allochthonous, and does not reflect the fungus population itself. For only fragments of fungal filaments, which have been formed by shaking the soil with water and are too short to develop when plated out, may be brought into suspension, a portion of which is plated out. Longer pieces of mycelium capable of development tend to remain on rapidly settling coarse soil particles, to which they are attached firmly.

2. By the use of a metal cap which is provided with a boring tube at the centre and with which the objective of the microscope is equipped, the mycelium can be picked out of the soil and cultivated on agar media, whereby specific determination of the mycelium is feasible. This method, if not always successful in isolating fungal mycelia directly from the soil, throws light to some extent on their occurrence in the soil.

3. It is not likely that WAKSMAN's direct inoculation method to prove the presence of mycelia in soil is invariably valid.

4. The specific gravity of mycelium is 1.121 in *Penicillium digitatum*, and 1.131 in *Trichoderma koningi* and *Alternaria* sp.

College of Arts and Science, Faculty of Education, Tôhoku University, Sendai.